Their relationships to bartonellae and other blood parasites and the peculiar type of infection which they produce should furnish interesting problems for future investigation.

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## *MECHANISM OF BIOLOGICAL NITROGEN FIXATION. VI. INHIBITION OF AZOTOBACTER BY HYDROGEN**

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Communicated January 27, 1941

A fundamental question in biological nitrogen fixation is whether the mechanism of fixation by the free-living *Azotobacter* is essentially the same as that of the symbiotic system (leguminous plants plus *Rhizobium*). Although some evidence exists in favor of an affirmative view, e.g., both are readily inhibited by combined nitrogen, convincing support has not been available. This deficiency arises mainly from a lack of suitable methods for defining the properties of the enzyme system concerned in the process, a deficiency that the physical-chemical studies of the past decade promise to overcome. Wilson and his collaborators,[1,2] for example, have demonstrated that molecular hydrogen acts as a specific, competitive inhibitor for nitrogen fixation by inoculated red clover plants. Since this reaction of $H_2$ with an enzyme system is quite unusual, a like demonstration with *Azotobacter* spp. should provide impressive evidence that the symbiotic and asymbiotic nitrogen fixation systems are essentially identical. Such a conclusion has important implications for studies on the mechanism of the biological fixation processes because, in addition to furnishing information on the nature of the responsible enzyme system, it establishes that results obtained with either type of fixation system are applicable to both.

In view of the importance of this conclusion it is desirable that the evidence in support be based on as many different types of observations as possible. The results discussed in this paper have been obtained in four types of experiments involving both macro and micro determinations made with representative strains of three species of *Azotobacter*. It is emphasized

that the experiments cited are only examples of many which have been made in confirmation and extension of this work.

*Methods.*—Cultures of *Az. vinelandii*, *Az. chroococcum* and *Az. agile* were grown on the following nitrogen-free media: $K_2HPO_4$, 0.8 g.; $KH_2PO_4$, 0.2 g.; $MgSO_4 \cdot 7H_2O$, 0.2 g.; NaCl, 0.2 g.; $CaSO_4 \cdot 2H_2O$, 0.1 g.; Fe as iron humate, 4 mg.; Mo as $Na_2MoO_4$, 1 mg.; agar, 1.0 g.; sucrose, alcohol or mannitol, 10–20 g. per liter. All cultures were purified by repeated plating on the nitrogen-free media; throughout the experiments the cultures were tested for purity by staining (Gram), streaking on a peptone nutrient agar plate, and inoculating a peptone nutrient broth tube. Contamination may frequently be detected in these media since *Azotobacter* grows very poorly in the presence of peptone. The gas mixtures were prepared from cylinder gases passed through a purifying chain of $H_2SO_4$, alkaline $KMnO_4$, distilled water and sterile cotton. Unless otherwise noted, the gas mixture had the following composition: oxygen, 20 per cent; nitrogen, 30 per cent; argon, helium or hydrogen, 50 per cent. The results, however, were not dependent on the use of a gas mixture of this particular composition as will be shown in a forthcoming paper. Details of the technique used in the various types of experiments will be supplied under the appropriate heading.

*Macro Total Nitrogen Experiments.*—A stock culture of the organism was prepared by inoculating 900 ml. of the medium with 30 ml. of a 24-hour-old culture of the bacteria on the same medium. After a few hours' incubation, 100 ml. portions were aseptically transferred to sterile liter bottles in the center of which was a test tube (24 × 100 mm.) containing 10 ml. of 20 per cent KOH. Each bottle was closed with a sterile, gas-tight rubber stopper through which passed a gas inlet tube that terminated inside the KOH "well." A sterile cotton filter in the tube also aided in preventing contamination from the in-coming gas. The desired gas mixture was prepared by drawing 18.4 in. of vacuum on the culture, then adding 3.6 in. of $O_2$ and the remaining gas to atmospheric pressure. The inlet tube was then connected to a manifold outlet by way of a water seal to prevent the atmospheres in the different bottles from mixing; the manifold was in turn connected with a reservoir of pure oxygen. At the start of an experiment the manifold and water seals were flushed with oxygen from the reservoir; during the test $CO_2$ from respiration by the bacteria was absorbed in the KOH with a consequent pressure deficit which drew in pure oxygen to replace that used in the bottle. Analyses showed that the system maintained the $O_2$ at 20 ± 1 per cent and that the $CO_2$ tension did not exceed 0.5 per cent. In some experiments no "inert" gas was supplied, but instead the cultures were grown under a total pressure of 0.5 atm.; in such cases the oxygen was supplied through a trap of mercury 15 inches high.

Results from four experiments given in table 1 show that the final total

nitrogen fixed by two species of *Azotobacter* is significantly lower when 0.5 atm. of nitrogen is replaced with hydrogen but is unaffected if helium or argon is used.

*Macro Rate Experiments.*—As has been previously emphasized,[1, 2, 3] erroneous conclusions may result if based solely on analyses at the end of an experiment. Rate experiments were accordingly made in which periodic analyses depicted the effect of hydrogen during the course of the fixation. Data from such experiments (table 2) indicate that the observed effect of hydrogen does not depend on any particular time of analysis. If these data are plotted, it becomes evident that the inhibition by $H_2$ persists throughout the test and is not merely an initial effect which prolongs the lag phase.

TABLE 1

COMPARISON OF EFFECT OF HYDROGEN AND HELIUM ON NITROGEN FIXATION BY *Azotobacter* (FINAL HARVEST)

| EXPERIMENT | ATMOSPHERE | TIME IN HOURS | NO. OF SAMPLES | MEAN NITROGEN FIXED, MG./100 ML. | DIFF. NECESSARY FOR SIGNIFICANCE,* MG. |
|---|---|---|---|---|---|
| 1 | Air | | | 6.35 | |
| | Helium | 72 | 4 | 6.62 | 2.00 |
| | Hydrogen | | | 4.00 | |
| 2 | Air | | | 8.13 | |
| | Helium | 96 | 3 | 8.13 | 1.67 |
| | Hydrogen | | | 5.47 | |
| 3 | Air | | | 5.05 | |
| | Helium | 72 | 2 | 5.80 | 1.67 |
| | Hydrogen | | | 3.10 | |
| 4 | Air | | | 19.9 | |
| | Helium | 36 | 2 | 19.8 | 3.77 |
| | Argon | | | 22.3 | |
| | Hydrogen | | | 12.9 | |

Experiments 1–3: *Az. agile* on ethyl alcohol at 30°C.; 100 ml.

Experiment 4: *Az. vinelandii* on sucrose at 30°C.; 25 ml.

* $P_{0.05}$ calculated from analysis of variance. $pN_2$ = 0.3 atm., $pO_2$ = 0.2 atm. whenever helium, argon or hydrogen were used.

Theoretically, under optimum conditions, the fixation should proceed logarithmically. If the *log mg. N* is plotted against time in experiments 5–7, however, a straight line is not obtained, the final point being too low. Investigation revealed that if 25 ml. of culture were used instead of 100 ml., so that a thin layer resulted, fixation would be maintained at a logarithmic rate sufficiently high to fix 15–25 mg. of nitrogen per 100 ml. in 36–48 hours (see experiment 8). This point is illustrated in typical experiments shown in figure 1. The slope of the line obtained when *log mg. N* was plotted against *time* was determined by the method of least squares, and the first order velocity constant of fixation, $k = 2.303 \times$ slope,† was calculated.

The observed difference in the slopes of any two lines was tested for significance by the usual statistical procedure. Slopes significantly different from that of the air control were noted only when the abstracted nitrogen was replaced with hydrogen.

The data in table 2 and figure 1 demonstrate two other important results of replacing part of the nitrogen in the atmosphere with other gases: (1) the rate of assimilation in a given experiment is independent of whether

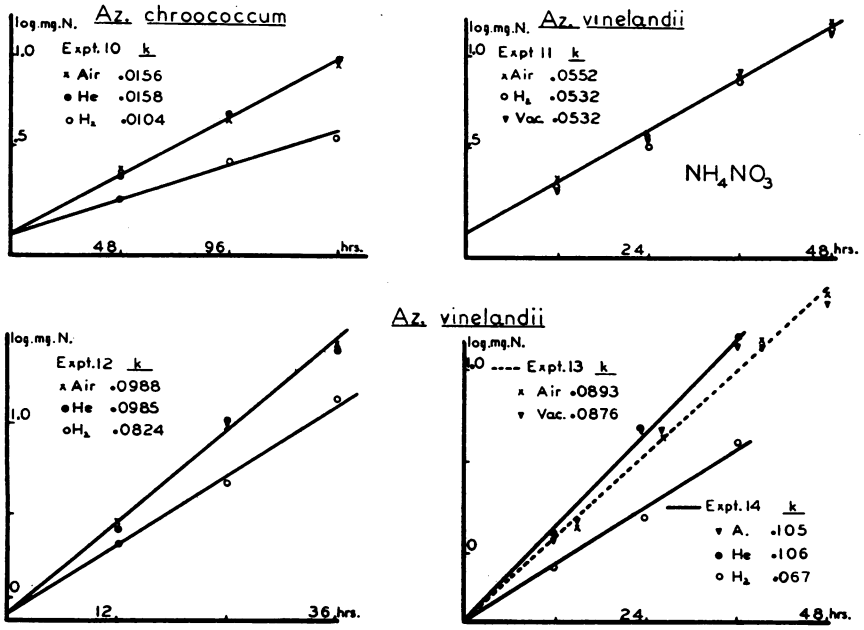


FIGURE 1

Rate of assimilation of free and combined nitrogen by macro cultures of *Azotobacter* in presence of argon, helium, hydrogen and a vacuum.

(In experiment 14 the $pN_2$ was 0.2 atm., the $pO_2$, 0.05 atm.; in all others the $pN_2$ was 0.3 atm., the $pO_2$, 0.2 atm. Vac. signifies that no inert gas was added; other symbols, the indicated gas to 1.00 atm.)

helium, argon or a vacuum is used in place of the 0.5 atm. of $N_2$ removed; (2) the assimilation of $NH_4NO_3$ is unaffected by $H_2$ in the atmosphere. This second point has already been reported by Burk using the Warburg microrespirometer for estimating fixation, but its demonstration for the experimental technique used here was believed desirable, since establishment of this fact leads to the conclusion that the effect of hydrogen is specifically concerned with the fixation reaction rather than a non-specific effect on general growth processes.

TABLE 2

INHIBITION BY HYDROGEN OF NITROGEN FIXATION BY *Azotobacter* (PERIODIC ANALYSES)

| EXPERIMENT | TIME IN HOURS | MG. N FIXED PER 100 ML. AIR | HELIUM | HYDROGEN |
|---|---|---|---|---|
| 5 | 24 | 1.50 | 1.52 | 0.82 |
| | 48 | 6.82 | 6.00 | 3.42 |
| | 72 | 7.50 | 8.12 | 5.70 |
| 6 | 24 | 1.25 | 1.10 | 0.80 |
| | 48 | 4.20 | 4.70 | 2.30 |
| | 72 | 7.35 | 6.13 | 4.17 |
| 7 | 48 | 1.83 | 1.95 | 1.42 |
| | | 1.80 | 1.60 | 1.33 |
| | 96 | 6.30 | 7.40 | 4.08 |
| | | 7.70 | 7.10 | 3.45 |
| | 144 | 10.25 | 10.65 | 5.50 |
| | | 9.45 | 10.50 | 6.25 |
| 8 | 15 | 3.0 | 3.0 | 2.5 |
| | 30 | 8.5 | 9.5 | 5.2 |
| | 45 | 19.7 | 18.6 | 11.9 |
| 9 | 17 | 8.04 | | 8.91 |
| $NH_4NO_3$ | 22 | 12.65 | | 12.95 |
| | 34 | 26.7 | | 26.7 |

Experiments 5 and 6: *Az. agile* on alcohol; 100 ml.
Experiment 7: *Az. vinelandii* on mannitol; 100 ml.
Experiment 8: *Az. vinelandii* on sucrose; 25 ml.; argon used instead of helium.
Experiment 9: *Az. vinelandii* on sucrose; 25 ml.; 28.2 mg. $NH_4NO_3$–N/100 ml.

*Microrespiration Experiments.*—The technique was essentially that of Burk[3] who first demonstrated that nitrogen fixation by *Azotobacter* might be indirectly estimated by observing the increase in the rate of oxygen uptake with time. Results from typical experiments are given in figure 2. When the *log rate $O_2$ uptake* (in $\mu l$/hr.) is plotted against *time*, a straight line results whose slope can be accurately determined by statistical means. As in the macro total nitrogen experiments, values of $k$ calculated from the slopes differed significantly only for the cells fixing nitrogen in the presence of hydrogen. In contrast, $k$ values for assimilation of $NH_4NO_3$ were identical within experimental error irrespective of the gas used to replace the abstracted nitrogen.

The underlying assumption of the microrespiration method is that increase in uptake of oxygen is a linear function of nitrogen fixation. In order to verify this, micronitrogen determinations were periodically made on the contents of a series of replicate Warburg flasks during some of the respiration trials. One such experiment is illustrated in figure 2. Although the $k$ values based on the micronitrogen determinations were definitely lower than those based on oxygen uptake the conclusions to be drawn were unaltered: nitrogen fixation was decreased if hydrogen replaced a

portion of the nitrogen in the atmosphere, but no decrease was observed if the replacing gas was helium.

*Turbidity Measurements.—Azotobacter* was grown in Thunberg tubes modified by Tam and Wilson[4] to allow measurement of turbidity in the Evelyn electrophotometer. The outlets of each series of triplicate cultures were connected with a water seal leading to the oxygen reservoir; KOH for absorption of evolved $CO_2$ was placed in the hollow stoppers. Readings were made every 12 hours; when the cell densities corresponding to the

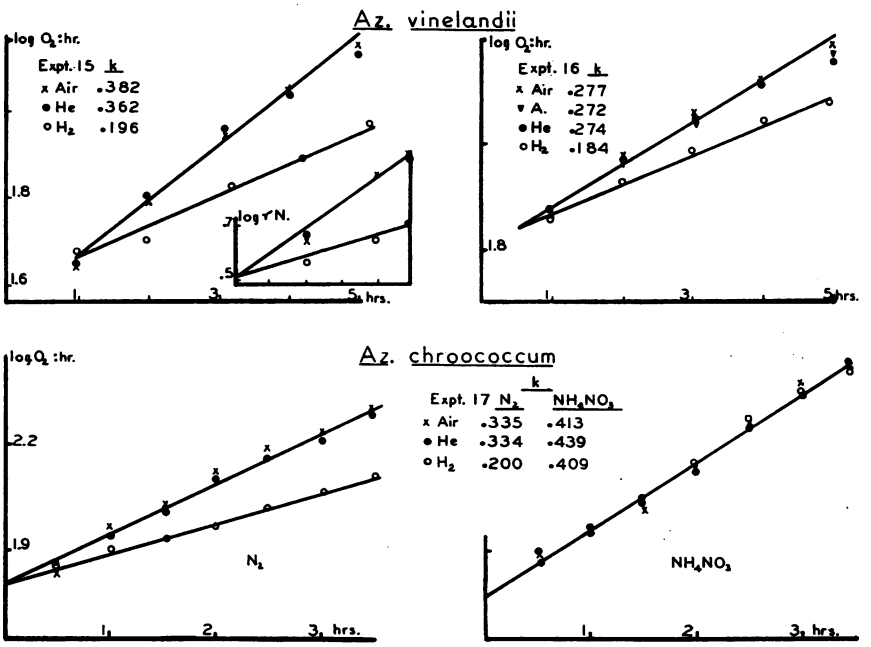


FIGURE 2

Increase in respiration rate of *Azotobacter* cultures growing on free and combined nitrogen in presence of argon, helium and hydrogen.

(Points shown are averages of 2 or 3 replicate cultures; $k$ values calculated from individual determinations).

readings were plotted on semilogarithmic paper, a straight line resulted. The following $k$ values were obtained with *Az. agile* on ethyl alcohol: air, 0.0222; helium, 0.0233; hydrogen, 0.0187. In another experiment *Az. vinelandii* on sucrose gave these values for $k$: air, 0.0448; helium, 0.0444; hydrogen, 0.0363. Statistical tests demonstrated that in each experiment the values for air and helium were the same within experimental error but that for hydrogen differed significantly from the others.

*Summary and Conclusions.*—Results from four types of experiments, involving both macro and micro estimation of nitrogen fixation by three

species of *Azotobacter*, consistently indicate that $H_2$ acts as a specific inhibitor of the fixation reaction in these species. *Azotobacter* cultures grown in an atmosphere in which the $pN_2$ is reduced to 0.3 atm., the $pO_2$ kept at 0.2 atm. and the abstracted $N_2$ either unreplaced or replaced with helium or argon, fix atmospheric $N_2$ at the same rate as that observed with cultures grown in air. If, however, $H_2$ is used to replace the $N_2$, a significant decrease is observed in both rate and extent of fixation. Since the symbiotic nitrogen fixation system of red clover responds to $H_2$ in the atmosphere in essentially the same manner, it is concluded that the mechanism of nitrogen fixation by the symbiotic system is similar, if not identical, with that of the fixation system in the free-living *Azotobacter*.

* This research was supported in part by a grant from the Rockefeller Foundation. Technical assistance on the experiments was supplied in part by workers employed in the University's Works Progress Administration Natural Science Project.

† A $k$ value of 0.1 means that the nitrogen content is doubled every 6.93 hours; one of 0.05, every 13.86 hours.

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## *NOTES ON BINARY STARS. IV. A SOURCE OF SPURIOUS ECCENTRICITY IN SPECTROSCOPIC BINARIES*

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Communicated February 5, 1941

*Introduction.*—Centrifugal and tidal forces cause a component of a binary star to deviate from a spherical shape, and to have a non-uniform surface brightness. The axial rotation of a deformed component, having a non-uniform surface brightness, contributes to its spectroscopically observed radial velocity. If the contribution is not allowed for, it must render the spectroscopic elements systematically erroneous, that are obtained in any of the usual manners from the radial velocity. Here we evaluate the contribution, to the radial velocity of a component of a spectroscopic binary star, made by the component's axial rotation in combination with its distortion and with its ordinary and "gravitational" darkening. Then we predict the effect of that contribution upon the spectroscopic orbital elements, as they are ordinarily deduced. We leave until another time the consideration of the contribution that arises from a combination of the axial